RESEARCH ARTICLE

Orally administered zingerone does not mitigate alcohol-induced hepatic oxidative stress in growing Sprague Dawley rat pups

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ABSTRACT

Neonatal alcohol exposure (NAE) can induce oxidative stress. We determined whether zingerone (ZO), a phytochemical with anti-oxidant activity, can mitigate the negative impact of neonatal alcohol-induced oxidative stress. Seventy ten-day-old Sprague-Dawley rat pups (35 male, 35 female) were randomly assigned and administered the following treatment regimens daily from postnatal day (PND) 12–21: group 1 – nutritive milk (NM), group 2 – NM +1 g/kg ethanol (Eth), group 3 – NM + 40 mg/kg ZO, group 4 – NM + Eth + ZO. Growth performance, blood glucose and plasma triglycerides (TGs), total cholesterol, HDL-cholesterol, leptin and insulin concentration were determined. Cytochrome p450E21(CYP2E1) and thiobarbituric acid (TBARS); markers of hepatic oxidative stress and catalase, superoxide dismutase (SOD) and total glutathione (GSH), anti-oxidant markers of the pups were determined. Oral administration of ethanol (NM + Eth), zingerone (NM + ZO) and combined ethanol and zingerone (NM + Eth + ZO) did not affect the growth performance and insulin and leptin concentration of the rats ($p > 0.05$). Ethanol significantly reduced plasma TGs concentration of female rats ($p = 0.04$ vs control). However, ethanol and/or its combination with zingerone decreased hepatic GSH ($p = 0.02$ vs control) and increased CYP2E1 ($p = 0.0002$ vs control) activity in male rat pups. Zingerone had no effect ($p > 0.05$ vs control) on the rats' CYP2E1, GSH, SOD and catalase activities. Neonatal alcohol administration elicited hepatic oxidative stress in male rat pups only, showing sexual dimorphism. Zingerone (NM + ZO) prevented an increase in CYP2E1 activity and a decrease in GSH concentration but did not prevent the alcohol-induced hepatic oxidative stress in the male rat pups.

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Zingerone; alcohol; oxidative stress; rat pups

Introduction

Alcohol consumption during pregnancy affects the mother and the developing fetus (Caputo *et al*. 2016). The umbrella term, fetal alcohol syndrome disorders (FASD), encompasses fetal alcohol syndrome (FAS), partial FAS, alcohol-related neurodevelopmental disorders and alcohol-related congenital disabilities (Caputo *et al*. 2016, Lange *et al*. 2017). Fetal alcohol syndrome is the most severe form of FASD (Caputo *et al*. 2016). In the general population, among every 13 pregnant women who consume alcohol, one is likely to have a child with FASD (Lange *et al*. 2017). FASD is avoidable if pregnant women abstain from excessive alcohol consumption. Studies indicate that women who consume alcohol during pregnancy are likely to continue indulging in the habit during lactation (Tran *et al*. 2015). Although alcohol consumption during breastfeeding may not directly cause FASD, it can have detrimental effects on the child and possibly impact health later in life (Anderson 2018).

Breastmilk is nutritional for babies such that exclusive breastfeeding is recommended for the first six months; after that, solid foods can supplement breastfeeding until the infant is two years old (WHO 2009). Studies have shown that alcohol can quickly move into breastmilk via passive diffusion (D'Apolito 2013). Alcohol reaches the breastmilk 0.5–1 hr after the mother has consumed it (Mennella 1998). About 95% of the mother's consumed dosage gets to her blood, and this closely parallels the amount of alcohol that enters the breastmilk (Anderson 2018). However, only 5–6% of the maternal blood alcohol level gets to the breastfeeding infants (Mennella 1998). Unfortunately, in newborns, the liver has not developed the capacity to metabolize and detoxify alcohol, thus increasing the chances of oxidative stress (Lavoie and Tremblay 2018). Several studies identify oxidative stress as a pre-eminent factor in ethanol teratogenicity (Bhatia *et al*. 2019). Three major pathways are involved in ethanol-induced oxidative stress, namely mitochondrial electron transport chain malfunction and increased activities of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the cytochrome P450 subfamily 2E1 (CYPE21) (Bhatia *et al*. 2019). These pathways generate reactive oxygen species, which, when in excess, lead to toxic effects such as increased lipid peroxidation, inactivation of enzymes, induction of DNA mutations and destruction of cell membranes (Bhatia *et al*.

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2019). Excessive generation of reactive oxygen species overwhelms an organism's anti-oxidant system, leading to oxidative stress and organ damage (Varga *et al*. 2017). Alcohol's effect on the developing fetus is a major area for research focus (Caputo *et al*. 2016). However, data on the effects of maternal alcohol consumption on offspring during lactation is scarce. It is important to note that the lactation period can have independent but equally significant impacts on metabolic programming (Ellsworth *et al*. 2018).

Several studies have shown that the administration of phytochemicals with anti-oxidant activity during the suckling period can protect against oxidative stress-related conditions (Lembede *et al*. 2018, Nyakudya *et al*. 2019). Zingerone, a phytochemical derived mainly from ginger (Ahmad *et al*. 2015), possesses anti-hyperlipidemic, anti-diabetic, anti-oxidant and anti-inflammatory properties (Ahmad *et al*. 2015, 2018, Cui *et al*. 2018). Based on the aforementioned pharmacological properties of zingerone, we determined whether zingerone can protect against alcohol-induced oxidative stress and metabolic effects in rat pups.

Materials and methods

Study site and ethics

All protocols and experimental procedures were approved by the Animal Research Ethics Committee of the University of Witwatersrand (Ethical clearance number: 2019/10/57/B). The study complied with accepted laboratory animal use and care principles stipulated in the South African National Standard (SANS 10386:2008) and the Animals Protection Act, 1962: Act No. 71. The study was undertaken at the Wits Research Animal Facility (WRAF)

Animal housing

This experiment used seventy (35 male and 35 female) 10-day-old Sprague Dawley rat pups obtained from 7 dams (litter 8–12). The pups were allowed to nurse freely from their respective dams throughout the suckling period from postnatal day (PND) 1 to 21. Dam and litter were kept in a temperature-controlled room (24 ± 2 °C) with a 12/12 h light-dark cycle with the light on from 7:00 h to 19:00 h. The animals were housed in acrylic cages with wood shavings for bedding.

Study design

Rat pups were assigned into four groups as follows: group 1-nutritional milk (NM), group 2 – NM + 1 g/kg of alcohol (Eth), group 3 – NM + 40 mg/kg of zingerone (ZO), and group 4 – NM+ 1 g/kg of alcohol (Eth) + 40 mg/kg of zingerone (ZO). Nutritive milk [Kitty milk (V 16752 Act 36/1947), Kyron Labs; Johannesburg, South Africa] was used as a vehicle for alcohol and zingerone. Supplementary data (S1–S6) provides a comparative analysis of using water or nutritive milk as a control. There was no considerable difference between using water or milk on the rats' organ morphometry and metabolic effects; thus we used the nutritive milk as control. After two days of habituation, the pups were administered via orogastric gavage their respective treatment regimens at a dose of 10 mL/kg between 9:00 am and 11:00 am daily from postnatal (PND) 12–21. PND10–21 coincides with the neonatal suckling period, a critical window of developmental plasticity in rats (Picut *et al*. 2015). Zingerone [Sigma-Aldrich (W312401); Johannesburg, South Africa] dosage used in the current study is similar to a previously described study by Muhammad *et al*. (2021). The alcohol (ACE; Johannesburg, South Africa) concentration used in this study parallels maternal blood alcohol and does not cause neurological effects (West *et al*. 1989, Anderson 2018). The alcohol concentration was estimated based on body mass and the human to rat dose conversion ratio (human: rat) of 1:6.17 as previously done (Reagan-Shaw *et al*. 2008).

Terminal procedures, sample collection and processing

On PND 22, the terminal body masses of the rat pups were measured using a calibrated electronic scale (Snowrex, Johannesburg, South Africa). After that, the rat pups were euthanized using an overdose (150 mg/kg) of sodium pentobarbital (Eutha-naze®, Bayer, Johannesburg, South Africa). Before the intraperitoneal injection of euthanaze, a drop of blood drawn via pinprick on the tail vein was used to determine blood glucose with a calibrated blood glucose meter (Contour Plus Bayer®, Johannesburg, South Africa). The blood was drawn via cardia puncture with a 21 G syringe into heparinized tubes and then centrifuged at 3000 × *g* for 15 mins to harvest plasma which was then stored at −80 °C till used for biochemical assays. Following blood collection, the liver and pancreas were dissected and weighed using an electronic balance (Snowrex, Johannesburg, South Africa). Each liver was then rinsed in cold saline, divided into two samples and then frozen and stored at −20°C for the determination of liver triglycerides and anti-oxidants assays.

After harvesting plasma, the right hind-limb tibia of each rat was dissected from the carcass and de-fleshed. The de-fleshed tibiae were then dried to a constant mass in an oven (Salvis®, Salvis Lab, Switzerland) at 50oC for six days. The dry mass of the tibiae was measured with an electronic scale (Presica 310 M, Presica Instruments AG, City, Switzerland) and the length with digital calipers (KTV 150 digital caliper, Elandsfontein, South Africa). The mass to length ratio of the tibiae was computed using the formula given by Seedor *et al*. (1991): tibiae density (mg/mm) = dry mass of bone (mg)/bone length (mm).

Liver tissue homogenization

Ten per cent of the liver sample was minced and homogenized in phosphate buffer (0.1 M, pH = 7.4) with an ultra turrax homogenizer (T-25 basic, Janke & Kunkel Ultra Turrax, Germany). The resultant homogenate was centrifuged at 3000 × *g* for 60 mins at 4^0 C. The harvested supernatant was used to determine hepatic oxidative stress, anti-oxidant enzyme activities and triglyceride content.

Determination of hepatic CYP2E1 concentration

Rat-specific CYP2E1 ELISA kits (Elabscience®, Wuhan, Hubei Province, China) were used to determine the hepatic CYP2E1 concentration (sensitivity range: 3.13–200 ng/mL). The test employed a sandwich ELISA principle. The assay was performed following the manufacturer's instructions. The optical density of the resulting reaction was measured at 450 nm on a microplate reader (Thermo Fisher Scientific Inc, Finland), and the sample concentrations were extrapolated from the standard curve.

Determination of hepatic thiobarbituric acid

Thiobarbituric acid (TBARS) concentration in the liver homogenate was estimated by the method of Niehaus and Samuelsson (1968). Briefly, 0.5 mL of the liver tissue homogenate sample was diluted with 0.5 mL of distilled water, after which 2.0 mL of the working reagent (TBA-TCA-HCl in a ratio of 1:1:1) was added. The mixture was boiled for 15 mins and allowed to cool on ice for 5mins. It was then centrifuged, and the supernatant was obtained. The absorbance of the supernatant was read spectrophotometrically (Beckman Coulter, USA, California) at 532 nm.

Determination of catalase activity

Liver catalase was estimated using the method described by Sinha (1972). To 0.1 mL of liver tissue homogenate in a glass test tube, 0.90 mL of phosphate buffer and 0.4 mL of H_2O_2 were added. The reaction was arrested at 15, 30, 45 and 60 seconds with 2 mL of dichromate acetic acid solution. The reaction was stopped by immersing the test tubes containing the reactants in a water bath with boiling water for 10 mins and cooled on ice. The color developed was measured on a spectrophotometer (Beckman Coulter, USA, California) at 610 nm. Catalase activity was expressed as μmoL of H_2O_2 consumed/min mg of protein for tissue.

Determination of superoxide dismutase activity and glutathione protein concentration

Hepatic superoxide dismutase (SOD) activity and total glutathione (GSH) protein were determined with biochemical assays kits (Elabscience®, Rat ELISA kit, Wuhan, Hubei Province, China) following the manufacturer's instructions. SOD activity was measured by the water-olubletetrazoliumsalt (WST-1) method. Xanthine oxidase (XO) catalyzes WST-1 and reacts with O^{2-} to generate a water-soluble formazan dye. Hepatic SOD inhibits the disproportionation of superoxide anions; thus, there is a negative correlation with the amount of formazan dye formed. SOD activity was determined by the colorimetric analysis of WST-1 products developed at 450 nm on a microplate reader (Thermo Fisher Scientific Inc, City, Finland).

Total protein was estimated by the Bradford method (Bradford 1976). Briefly, 5 μL of the liver homogenate was added to 250 μL of sigma's Bradford reagent (B6916; Sigma, Germany). The reactants were mixed thoroughly and incubated for 10mins at room temperature. The plate was read at 595 nm on a microplate reader (Thermo Fisher Scientific Inc, Finland).

For the determination of total GSH concentration, oxidized glutathione is reduced by glutathione reductase in the sample, and the reduced GSH reacts with 5,5′-dithio-bis (2-nitrobenzoic acid) to produce oxidized glutathione (GSSG) and yellow 2-nitro-5-mercapto-benzoic acid (TNB). The amount of total glutathione determines the amount of yellow 2-nitro-5-mercapto-benzoic acid (TNB) formed. This was measured colorimetrically at a wavelength of 412 nm on a microplate reader (Thermo Fisher Scientific Inc, City, Finland).

Determination of liver triglyceride content

With the liver homogenate obtained, triglyceride (TG) concentration was assayed using kits from Elabscience®, Wuhan, Hubei Province, China) following the manufacturers' instructions. In summary, 2.5 μL of liver homogenate was diluted with 2.5 μL of distilled water and mixed with 250 μL of TG reagent. The mixture was incubated at 37 °C for 10mins, and the absorbance was read on a microplate reader (Thermo Fisher Scientific Inc, City, Finland) at 510 nm. Hepatic TG was expressed as gprot/L.

Determination of plasma lipid profile

Plasma triglyceride, total cholesterol and high-density lipoprotein cholesterol concentration were determined using colorimetric assay kits as per the manufacturer's instruction (Elabscience®, Wuhan, Hubei Province, China). Low-density lipoprotein cholesterol concentration was estimated using the Friedewald equation: LDL-cholesterol = (TC-HDL-C)-(TGs/5) (Knopfholz *et al.* 2014).

Determination of plasma leptin and insulin concentration

Rat-specific insulin (INS), leptin (LEP) ELISA kits (Elabscience®, Rat INS (Insulin) ELISA kit, Wuhan, Hubei Province, China) were used to determine the plasma insulin and leptin concentration, respectively, according to the manufacturer's instructions. Insulin resistance was computed based on the homeostasis model assessment of insulin resistance (Matthews *et al.* 1985): HOMA-IR = fasting plasma insulin (μU/mL) × fasting glucose (mmol/L)/22.5

Data analysis

GraphPad Prism 8 software was used to analyze the data. Data were expressed as mean ± standard deviation. A one-way ANOVA was used followed by the multiple-comparisons Tukey *post hoc* test to compare the means. Statistical significance was considered when $p < 0.05$.

Results

Effects on growth performance

Figure 1 shows the effect of the treatment regimens on the body mass of the rat pups over the experimental period. There was no statistically significant difference in the

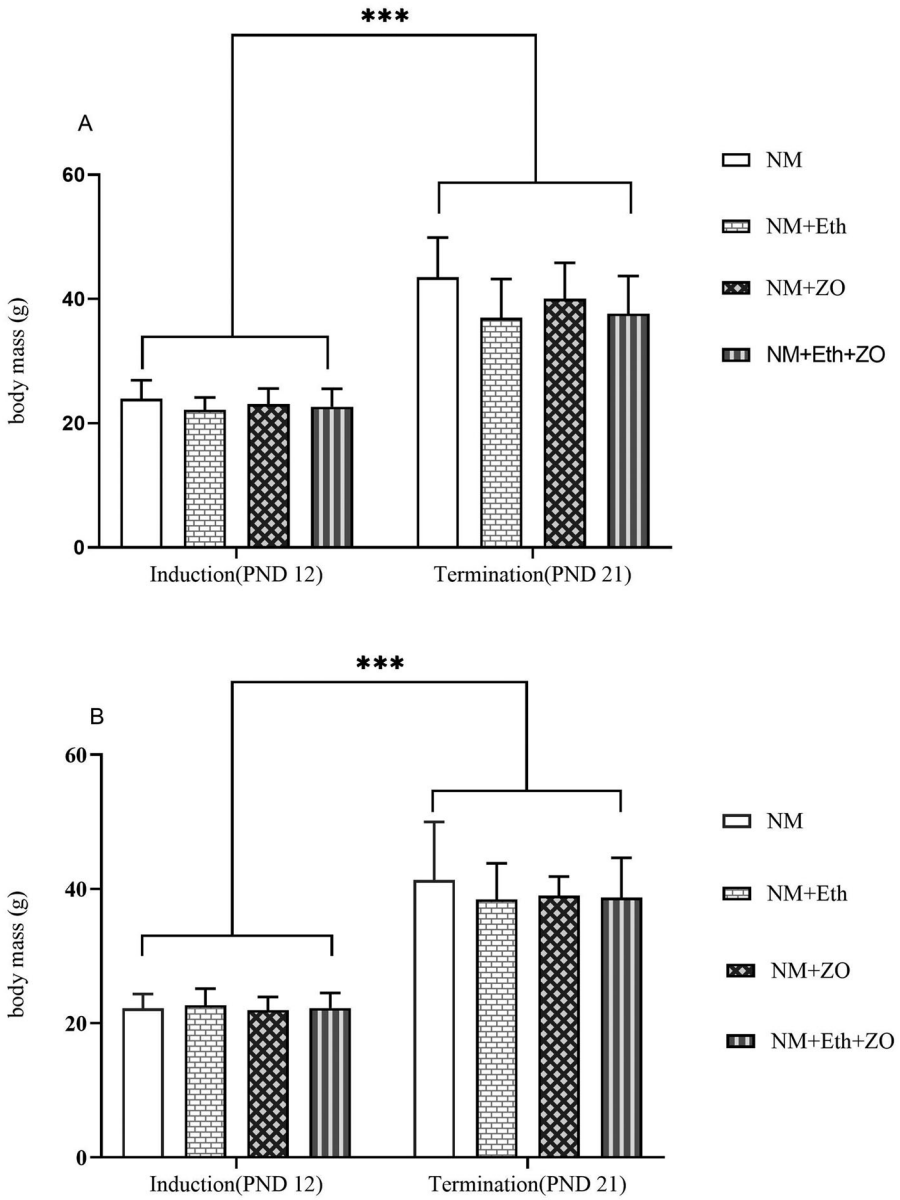

Figure 1. Effect of neonatal oral administration zingerone on the terminal body masses of male (A) and female (B) rat pups exposed to alcohol. Data presented as mean ± SD. ***$p < 0.01$ when induction body mass is compared to terminal body mass. NM = gavaged with 10 mL/kg of nutritive milk: NM + Eth = gavaged with 1g/kg of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg in nutritive milk; NM + Eth + ZO = gavaged with 1g/kg of alcohol + 40 mg/kg of zingerone in nutritive milk. $n = 8–10$ per treatment.

induction [$p = 0.925$ (female); $p = 0.704$ (males); Figure 1] and terminal [$p = 0.772$ (females); $p = 0.148$ (males); Figure 1] body masses of the rat pups across treatment groups in female and male rat pups. However, all rat pups grew significantly ($p < 0.0001$; Figure 1).

The empty carcass of the females was similar across treatment groups ($p > 0.539$; Table 1). Similarly, there was no significant difference in the empty carcass of the male rat pups ($p = 0.144$; Table 1). Neonatal exposure to alcohol, zingerone and or a combination of alcohol and zingerone had no effect on the tibiae mass, length and mass/length ratio of the male and female rat pups ($P > 0.05$; Table 1).

Effects on gross liver and pancreas morphometry

Absolute and relative liver masses were similar across the treatment groups for males and females ($p > 0.05$; Table 2). The absolute pancreas mass was significantly different in male rat pups ($p = 0.012$; Table 2): alcohol (NM + Eth) significantly reduced the absolute pancreas mass ($p = 0.010$ vs control), but pancreata mass relative to tibia length was similar in the male and female rat pups across treatment regimens ($p > 0.05$; Table 2).

Effects on oxidative stress and anti-oxidants

In the females, there was no statistical significance across treatment for oxidative stress biomarkers; CYP2E1 ($p = 0.126$; Supplementary Figure 1) and TBARS ($p = 0.946$; Supplementary Figure 2). Similarly, treatments had no effect on the anti-oxidants catalase ($p = 0.934$; Supplementary Figure 2), SOD ($p = 0.446$; Supplementary Figure 2) and GSH ($p = 0.074$; Supplementary Figure 2) in the female rat pups.

Table 1. The effect of neonatal oral administration of zingerone on long bone indices and empty carcass in suckling pups exposed to alcohol.

Parameter	Sex	NM	NM + Eth	NM + ZO	NM + Eth + ZO
Tibia mass (mg)	Female	46.0 ± 13.3	40.6 ± 6.4	42.3 ± 4.5	41.1 ± 6.6
	Male	48.9 ± 9.8	41.6 ± 5.3	43.0 ± 5.2	41.8 ± 5.3
Tibia length(mm)	Female	15.4 ± 1.2	15.2 ± 0.7	15.3 ± 0.6	15.1 ± 0.6
	Male	15.9 ± 1.3	14.9 ± 1.1	15.0 ± 0.8	14.9 ± 0.5
Tibia mass/length(mg/mm)	Female	2.9 ± 0.6	2.7 ± 0.3	2.8 ± 0.2	2.7 ± 0.3
	Male	3.1 ± 0.4	2.8 ± 0.3	2.86 ± 0.20	2.8 ± 0.3
Empty carcass	Female	32.8 ± 7.1	30.2 ± 4.1	30.2 ± 3.8	29.9 ± 4.6
	Male	33.5 ± 5.4	28.7 ± 4.6	30.8 ± 4.3	28.3 ± 5.5

Data presented as mean ± SD. $p > 0.05$. NM = gavaged with 10 mL/kg bwt of nutritive milk: NM + Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM + Eth + ZO = gavaged with 1 g/k bwtg of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. $n = 8$–10 per treatment.

Table 2. The effect of neonatal oral administration of zingerone on liver and Pancreas weight in suckling rat pups exposed to alcohol.

Parameter	Sex	NM	NM + Eth	NM + ZO	NM + Eth + ZO
Liver mass (g)	Female	1.80 ± 0.37[a]	1.83 ± 0.23[a]	1.81 ± 0.30[a]	1.87 ± 0.23[a]
	Male	1.19 ± 0.21[a]	1.23 ± 0.14[a]	1.91 ± 0.21[a]	1.26 ± 0.24[a]
Liver rTL(g/mm)	Female	1.93 ± 0.40[a]	1.70 ± 0.35[a]	1.83 ± 0.31[a]	1.26 ± 0.37[a]
	Male	1.18 ± 0.15[a]	1.11 ± 0.07[a]	1.22 ± 0.19[a]	1.10 ± 0.18[a]
Pancreas (g)	Female	0.21 ± 0.04[a]	0.21 ± 0.06[a]	0.22 ± 0.08[a]	0.20 ± 0.06[a]
	Male	0.24 ± 0.05[a]	0.12 ± 0.04[b]	0.21 ± 0.06[ab]	0.18 ± 0.04[ab]
PancreasrTL(g/mm)	Female	0.14 ± 0.03[a]	0.14 ± 0.04a	0.15 ± 0.04[a]	0.12 ± 0.05[a]
	Male	0.13 ± 0.06[a]	0.10 ± 0.03[a]	0.14 ± 0.03[a]	0.14 ± 0.04[a]

Data presented as mean ± standard deviation. *P* values were set at $p < 0.05$. [ab] = within row means with different letters significantly different at $p < 0.05$. NM = gavaged with 10 mL/kg bwt of nutritive milk; NM + Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM + Eth + ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. Liver rTL = liver mass relative to tibial length, PancreasrTL = pancreas mass relative to tibial length. $n = 8$–10 per treatment.

In male rat pups, alcohol (NM + Eth) significantly increased hepatic CYP2E1 concentration ($p = 0.0002$ vs control; Figure 2A). The administration of zingerone (NM + ZO) resulted in a similar CYP2E1 concentration ($p = 0.188$; Figure 2A) to that of rats administered the control (NM). Co-administration of Eth and ZO increased ($p = 0.047$ vs control) CYP2E1 concentration. Catalase and SOD activity were similar in the rat pups across treatment regimens ($p > 0.05$; Figure 3A and B). Neonatal oral administration of alcohol (NM + Eth) significantly decreased ($p = 0.041$ vs control; Figure 3C) GSH concentration. Additionally, GSH concentration was significantly higher in the ZO group (NM + ZO) compared to ethanol group (NM + Eth) ($p = 0.003$; Figure 3C), but the ZO did not mitigate the alcohol-induced decrease in combined alcohol and ZO (NM + Eth + ZO) group ($p = 0.717$ vs control). Treatment regimens had no effects on the TBARS concentration of the male rat pups ($p = 0.371$; Figure 3C).

Effects on plasma lipids, glucose, leptin and insulin concentration, hepatic triglycerides and HOMA-IR

Treatment regimens had no effect on the lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol) of both female and male rat pups (Table 3; $p > 0.05$), except for plasma TG. In female rat pups, when compared to control (NM), the oral administration of alcohol (NM + Eth) significantly reduced ($p = 0.044$; Table 3) TG concentration. TG concentration was not significantly different in the male rat pups ($p = 0.202$; Table 3). Neonatal oral administration with ZO ($p = 0.305$; Table 3) and combined Eth and ZO ($p = 0.995$; Table 3) did not affect plasma TG concentration.

Visceral fat mass relative to per cent body mass was not statistically different across treatment regimens for females ($p = 0.523$; Supplementary Figure 1B) and males ($p = 0.704$; Figure 2B). Liver TG concentration was similar in female and male rat pups ($p = 0.213$; Supplementary Figure 1C, $p = 0.612$; Figure 2C) across treatment regimens. Table 4 shows the effect of the treatment regimens on the rat pus' blood glucose, plasma leptin and insulin concentration and HOMA-IR. Neonatal oral administration of alcohol (NM + Eth), zingerone (NM + ZO) and combined alcohol and zingerone (NM + Eth + ZO) had no effect on the rat pups' blood glucose, plasma insulin and leptin concentration and HOMA-IR ($p > 0.05$; Table 4).

Discussion

In this study, we investigated the potential of neonatally administered zingerone to protect against the negative impact of neonatal alcohol-induced oxidative stress in male and female Sprague Dawley rats. We showed that neonatal alcohol exposure (NAE) in rat pups induces oxidative stress, which may have a long-lasting impact on metabolic profile in adulthood. A sexually dimorphic outcome was observed in response to NAE: alcohol-induced elevated CYP2E1 concentration and reduced total glutathione in male rat pups, while in female rat pups it elicited a reduction in plasma TG. Zingerone did not affect growth performance, hepatic CYPE21 concentration and triglycerides, plasma lipids and blood glucose metabolism. Still, zingerone failed to prevent the alcohol-induced CYP2E1 induction and reduced glutathione concentration.

In this study, body mass was unaffected by any treatment regimen. Body mass can be affected by hydration and feeding status; hence, other growth performance indicators are preferred (Sundström *et al.* 2014). However, we did not find

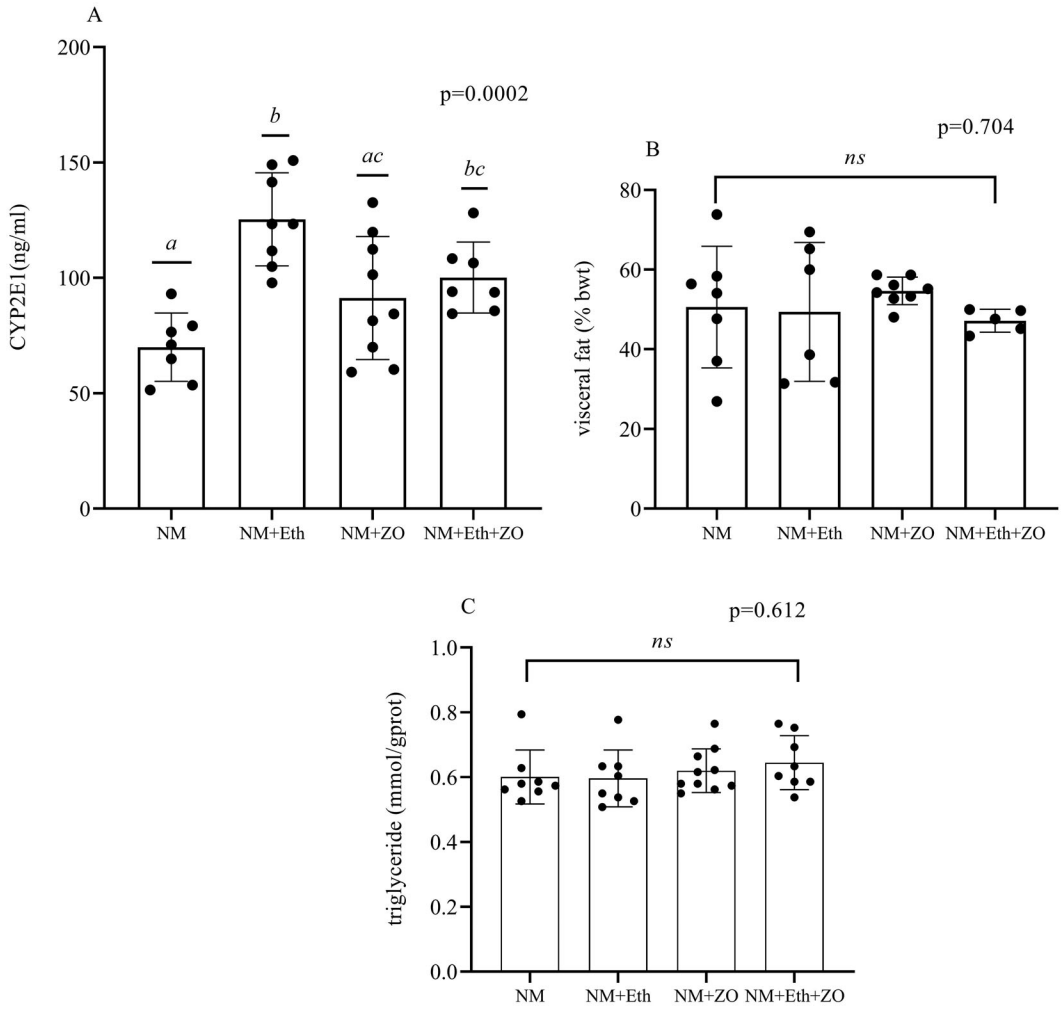

Figure 2. Effect of neonatal oral administration of zingerone on (A) liver cytochrome p450, (B) visceral fat mass (% b.wt) and (C) and triglycerides of male rat pups exposed to alcohol. Data presented as mean ± SD. ns=$p > 0.05$ when a statistical comparison is non-significant across treatment groups, ^{ab} = within row means with different letters significantly different at $p < 0.05$. NM = gavaged with 10 mL/kg of nutritive milk: NM + Eth = gavaged with 1g/kg of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40mg/kg in nutritive milk; NM + Eth + ZO = gavaged with 1g/kg of alcohol + 40mg/kg of zingerone in nutritive milk. $n = 8$–10 per treatment.

any significant effect of NAE on the empty carcass and long bone indices, indicating that NAE did not affect growth performance. In a comparable study in which rat pups received alcohol through breastfeeding, no change in body mass was recorded (Chen and Nyomba 2004). Contrary, prenatal alcohol exposure decreased body mass because of nutrient deprivation (de Freitas *et al*. 2014, Cheng *et al*. 2021). However, using rat pups that can suckle from their dams along with the intervention in this study did not affect caloric intake. Additionally, the ethanol was not administered to the dams. Ethanol administration in dams affects maternal care and decreases milk production (Pepino *et al*. 2007, Ponce *et al*. 2011). Furthermore, we observed that the rat pups opened their eyes by PND 15 and nibbled on the rat chow available to the dams; thus, in addition to maternal milk and nutritive milk, the pups also got some of the energy from the rat chow.

Zingerone did not affect body mass and empty carcass. Other studies have reported that ZO does not affect body mass in adult rats (Cui *et al*. 2018; Muhammad *et al*. 2021). Decreased bone mass is related to decreased bone formation due to reduced osteocalcin, IGF-1 and vitamin D (González-Reimers *et al*. 2005). However, ZO did not affect long bone indices, suggesting that ZO may have osteogenic properties as previously reported by Srinaath *et al*. (2019).

Nutrition and hepatic pathology affect liver mass; an increase in liver mass can result from a fatty liver, while a decrease can be due to undernutrition (Pandit and Gupta 2019). Since the liver is the center of ethanol metabolism, we examined its gross morphometry and possible oxidative damage. The absolute liver mass and its mass relative to the tibiae length regardless of treatment or sex were unaffected. This agreed with our earlier speculation that NAE and zingerone did not affect the nutritional intake of the rat pups. Malnutrition results in hypoleptinemia (Paillaud *et al*. 2022). However, leptin was also not affected by any of the treatment interventions.

Alcohol-treated male rat pups had an elevated CYPE21 concentration. Generally, CYP2E1 is induced during excess alcohol consumption, but other studies suggest that the microsomal ethanol-oxidizing system (MEOS) induced by CYP2E1 and catalase may be the primary ethanol metabolizing enzyme even at low ethanol concentration (Takagi *et al*. 1986, Peana *et al*. 2017). In support of our findings, Chhabra

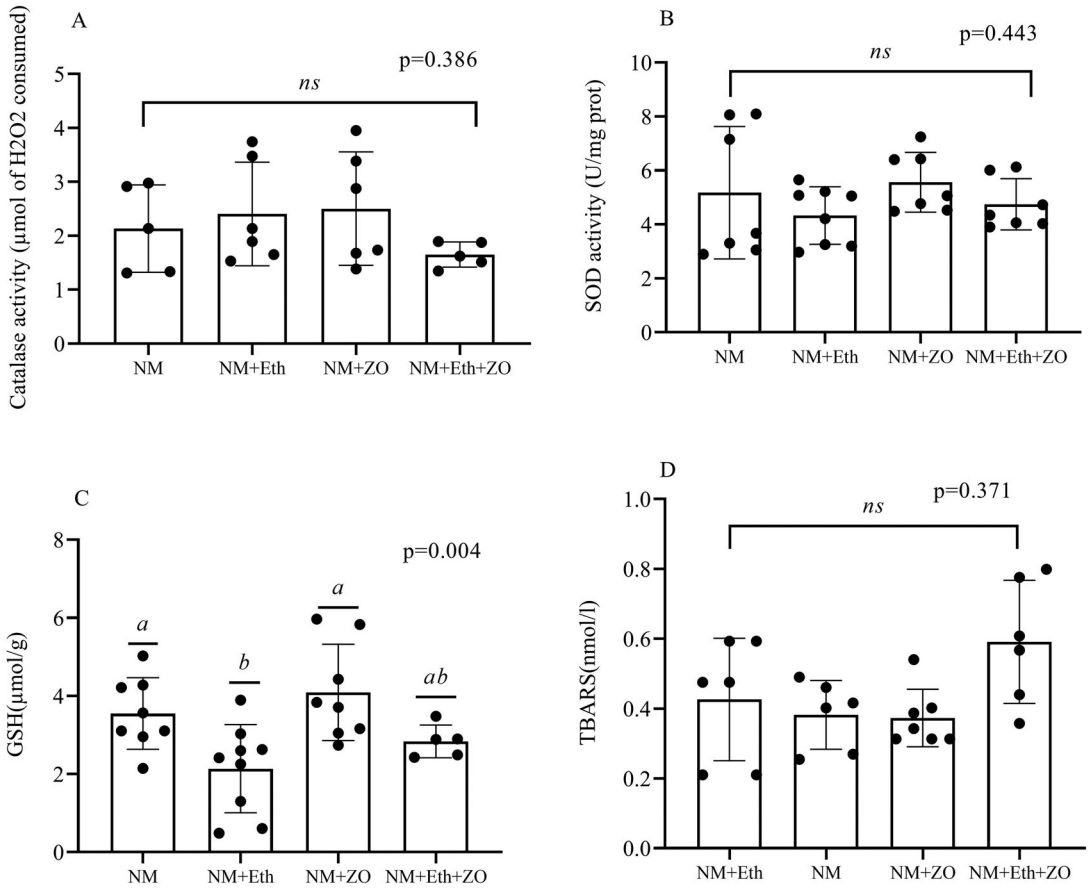

Figure 3. Effect of neonatal oral administration of zingerone on anti-oxidants (A) Catalase activity (B) Superoxide dismutase (C) Glutathione proteins and liver oxidative stress (D) TBARS in male rat pups exposed to alcohol. ns=$p > 0.05$ when the statistical comparison is non-significant across treatment groups. ^{ab} = within row means with different letters significantly different at $p < 0.05$. NM = gavaged with 10 mL/kg of nutritive milk: NM + Eth = gavaged with 1g/kg of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg in nutritive milk; NM + Eth + ZO = gavaged with 1 g/kg of alcohol + 40mg/kg of zingerone in nutritive milk. $n = 8$–10 per treatment.

Table 3. The effect of neonatal oral administration of zingerone on lipid profile in suckling rat pups exposed to alcohol.

Parameter	Sex	NM	NM + Eth	NM + ZO	NM + Eth + ZO
TG (mmol/L)	Female	0.85 ± 0.21^{a}	0.56 ± 0.15^{b}	0.69 ± 0.19^{ab}	0.84 ± 0.23^{ab}
	Male	0.88 ± 0.37^{a}	0.71 ± 0.13^{a}	0.68 ± 0.19^{a}	0.90 ± 0.34^{a}
HDL (mmol/L)	Female	1.63 ± 0.46^{a}	1.78 ± 0.44^{a}	1.62 ± 0.41^{a}	1.85 ± 0.38^{a}
	Male	1.68 ± 0.42^{a}	1.61 ± 0.42^{a}	1.66 ± 0.60^{a}	1.71 ± 0.51^{a}
TC (mmol/L)	Female	2.49 ± 0.76^{a}	2.35 ± 0.60^{a}	2.37 ± 0.65^{a}	2.62 ± 0.91^{a}
	Male	2.66 ± 0.91^{a}	2.34 ± 0.50^{a}	2.25 ± 0.39^{a}	2.87 ± 0.83^{a}
LDL (mmol/L)	Female	1.19 ± 0.96^{a}	1.82 ± 0.71^{a}	1.23 ± 0.45^{a}	1.38 ± 0.29^{a}
	Male	1.35 ± 0.54^{a}	1.19 ± 0.37^{a}	0.74 ± 0.32^{a}	1.43 ± 0.79^{a}

Data presented as mean ± standard deviation. P values was set at $p < 0.05$. ^{ab} = within row means with different letters significantly different at $p < 0.05$. NM = gavaged with 10 mL/kg bwt of nutritive milk: NM + Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM + Eth + ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. TG = Triglycerides; HDL-high density lipoprotein cholesterol; TC = Total cholesterol; LDL = Low density lipoprotein cholesterol.

et al. (1996) demonstrated that rat pups exposed to alcohol through their dams' milk induced liver CYP2E1; however, the induction was significantly high in male rat pups compared to female counterparts. The induction of CYP2E1 is associated with the generation of reactive oxygen species, which can induce oxidative stress (Bhatia *et al.* 2019).

In the current study, zingerone did not induce CYP2E1. The potent anti-oxidant and anti-inflammatory properties of ZO might have modulated the production of CYP2E1 (Bashir *et al.* 2021). The previous report demonstrated that pretreatment with ZO blunts elevation of CYP2E1 transcripts when exposed to Cisplatin (Cis) or γ-Irradiation (IR)-induced hepatotoxicity in adult male albino rats (Mohamed and Badawy 2019).

Hepatic catalase activity was similar in the rat pups across treatment regimens. This result was unexpected, especially in the alcohol-treated group, as catalase plays an accessory role in ethanol metabolism. However contradictory results concerning catalase activity in rat pups have been reported by Ojeda *et al.* (2009), who observed an increased activity, while de Freitas *et al.* (2014) reported a decrease in catalase activity. Our results suggest that catalase may not be actively involved in hepatic ethanol metabolism, as a previous study

Table 4. The effect of neonatal oral administration of zingerone on glucose and leptin in suckling rat pups exposed to alcohol.

Parameter	Sex	NM	NM + Eth	NM + ZO	NM + Eth + ZO
Glucose (mmol/L)	Female	5.74 ± 0.53	5.49 ± 0.39	5.25 ± 0.82	5.56 ± 0.80
	Male	5.41 ± 0.37	5.48 ± 1.27	5.90 ± 0.53	5.96 ± 0.99
Insulin (ng/mL)	Female	1.10 ± 0.47	1.29 ± 0.24	1.08 ± 0.36	1.26 ± 0.52
	Male	1.10 ± 0.32	0.99 ± 0.32	1.31 ± 0.17	0.95 ± 0.23
HOMA-IR	Female	0.37 ± 0.18	0.30 ± 0.10	0.30 ± 0.09	0.40 ± 0.18
	Male	0.35 ± 0.18	0.31 ± 0.15	0.44 ± 0.12	0.30 ± 0.13
Leptin (ng/mL)	Female	0.91 ± 0.21	0.88 ± 0.16	0.80 ± 0.12	0.90 ± 0.23
	Male	0.89 ± 0.27	0.90 ± 0.13	0.75 ± 0.25	1.05 ± 0.28

Data presented as mean ± standard deviation $p > 0.05$. NM = gavaged with 10 mL/kg bwt of nutritive milk: NM + Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM + ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM + Eth + ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. HOMA-IR = homeostatic model assessment of insulin resistance. $n = 8$–10 per treatment.

indicates that the pharmacokinetics of ethanol in liver cells does not involve catalase (Aragon *et al*. 1989).

In the current study, SOD activity was not affected by the neonatal treatments. This finding contradicts other studies (Cheng and Kong 2011, Kołota *et al*. 2019). However, the variance in our findings might be related to the age of the animals, as they used adult rats, unlike the neonate rats used in this study. Aging is associated with a decline in mitochondrion enzyme activities involved in scavenging free radicals (Kaplán *et al*. 2019). Related studies with rat pups that received ethanol during lactation also reported that NAE did not affect activity (Ojeda *et al*. 2009, de Freitas *et al*. 2014).

Our findings point to an alcohol-induced reduction in total glutathione (GSH) concentration in the male rat pups. Previous studies observed a decrease in hepatic glutathione peroxidase (GPx) and glutathione-S-transferase (Ojeda *et al*. 2009, de Freitas *et al*. 2014). Glutathione peroxidase activity correlates positively with GSH levels, as GSH serves as an essential cofactor for GPx activity. Alcohol inhibits the transport of cytosolic GSH into the mitochondrion leading to depletion and affecting the amount of glutathione needed to bind acetaldehyde (Fernández-Checa 2003). This allows for the formation of excessive amounts of free radical species, causing oxidative stress. We observed that alcohol induces a reduction in glutathione concentration in a sexually dimorphic manner as only males had a reduced concentration. This effect may be related to estrogens in females (Lavoie and Tremblay 2018); estrogens have been shown to induce glutathione metabolism and contribute significantly to sex specificity in oxidative stress because of it (glutathione) controls intracellular levels of peroxides, aldehydes and radicals (Lavoie and Tremblay 2018). It is noteworthy that both males and females are exposed to high levels of estrogen *in-utero*; however, there is a perinatal surge in testosterone in the first week of the rodent life (Dearden *et al*. 2018), thus reducing estrogen functionality.

Despite the above observation, there was no significant increase in the hepatic TBARS concentration in the alcohol-exposed pups. A previous study reported similar findings (de Freitas *et al*. 2014). In lipid peroxidation, free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially the C-C double bonds of polyunsaturated fatty acids (Ayala *et al*. 2014). Perhaps the small amount of fat content of the rat pups contributed to the non-detection of lipid peroxidation (Tavares do Carmo *et al*. 1999). However, we did not determine cholesterol oxidized products.

Orally administered zingerone only maintained anti-oxidant ability and prevented an elevation in CYP2E1 concentration comparable to the control. Zingerone is a polyphenol, and by its (ZO) redox properties can act as free radical quenchers, reducing agents, hydrogen donors, metal chelators, and decomposers of peroxides hence preventing oxidative stress (Bashir *et al*. 2021). However, zingerone combined with alcohol did not influence CYP2E1 and glutathione concentration. Aeschbach *et al*. (1994) demonstrated that although zingerone has anti-oxidant properties, its inhibitory action on oxidative stress is weak relative to other components of ginger like geraniol, and 6-gingerol. Meanwhile, studies have also reported that zingerone does not affect GSH and lipid peroxidation (Aeschbach *et al*. 1994, Eid *et al*. 2017). Additionally, besides the short duration of administration, the co-administration of alcohol with zingerone might have reduced the anti-oxidative potential of zingerone. Previous pre-clinical studies investigating the association between early-life and offspring metabolic health typically use a high dose [2 g kg^{-1} b.w. administered twice a day (Akison *et al*. 2019)] throughout gestation and during lactation. This likely increased blood alcohol availability and thus the pronounced metabolic effect observed in those studies. The low, acute dose model that we used in the current study mimics the amount of alcohol that would be consumed by the breastfeeding infants of lactating women who report drinking on average one or two standard drinks per day.

In this study, NAE had no effect on plasma insulin and insulin sensitivity of the rat pups. The low dose short-term alcohol administration used in this study possibly reduced insulin concentration and improved insulin sensitivity, particularly in females compared to males, as previously reported by other studies (Schrieks *et al*. 2015). Zingerone did not affect insulin concentration in the rat pups; however, in diabetic rats and humans, treatment with zingerone reduces insulin levels and maintains normal glucose concentrations (Shidfar *et al*. 2015, Cui *et al*. 2018). The anti-diabetic effect of zingerone is likely due to its ability to activate peroxisome proliferator-activated receptors (*PPARs)* expression (Chung *et al*. 2009), which play an essential role in glucose and lipid homeostasis (Hassan *et al*. 2021).

Alcohol-treated female rat pups had significantly reduced plasma triglycerides, similar to the study of Gårdebjer *et al*. (2018), but tissue TG was unaffected. It is possible that the fat was being redistributed to peripheral tissue resulting in hypotriglyceridemia as has been reported in adult rats that consumed alcohol (Steiner and Lang 2017). Plasma and tissue lipids were unaffected by zingerone treatment. Studies show that suckling rats have scanty adipose tissue thus the amount of free fatty acids and glycerol released into circulation is not

quantitatively relevant (Tavares do Carmo *et al*. 1999). Hence the effect of the interventions on plasma lipids cannot be considered relevant in rat pups.

In vitro studies show that ZO scavenges free radicals in a dose-dependent manner; the higher the dosage, the better its scavenging potential (Rao *et al*. 2009). Mani *et al*. (2016) administered 40 mg/kg zingerone to adult Wistar rats which were effective at potentiating the anti-oxidant system of the rats against alcohol-induced oxidative stress. This dosage of zingerone (40 mg/kg) was previously used in rat pups and this dosage was found to be effective at preventing the development of nonalcoholic fatty liver disease in adulthood (Muhammad *et al*. 2021). Zingerone has a pungent taste that can activate transient receptor potential cation channel subfamily V member 1 (TRPV1), the receptor for heat and pain (Komai *et al*. 2006). Thus, investigators were concerned that a higher zingerone dose might cause the rat pups gastric discomfort and consequently impact acceptability by the rat pups. As such we used 40 mg/kg as previously reported by other researchers conducting similar studies. Although a higher zingerone dosage might have been effective.

Conclusion

Due to reduced adiposity in suckling rat pups, the pups had stronger resistance to developing metabolic disorders associated with metabolic syndrome (Ghezzi *et al*. 2012). However, low-dose NAE induced hepatic oxidative stress via elevating CYP2E1 and decreasing GSH concentrations in males only, while orally administered zingerone did not affect hepatic CYPE21, anti-oxidant status and growth performance. Therefore, ZO can be administered like a safe alternative drug for oxidative-stress-related conditions in neonates and this may confer positive health status in adulthood.

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Data availability statement

Researchers may request data from the authors. Additional data is provided.

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